

Lipid Nanoparticles for Delivery of Therapeutic RNA Oligonucleotides

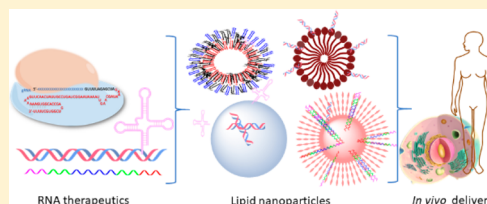
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ABSTRACT: Gene therapy is an exciting field that has the potential to address emerging scientific and therapeutic tasks. RNA-based gene therapy has made remarkable progress in recent decades. Nevertheless, efficient targeted delivery of RNA therapeutics is still a prerequisite for entering the clinics. In this review, we introduce current delivery methods for RNA gene therapeutics based on lipid nanoparticles (LNPs). We focus on the clinical appeal of recent RNA NPs and discuss existing challenges of fabrication and screening LNP candidates for effective translation into drugs of human metabolic diseases and cancer.

KEYWORDS: gene therapy, RNA, delivery, lipid nanoparticle (LNP), LNP fabrication, LNP screening



INTRODUCTION

RNA therapeutics is a broad group of RNA oligo- and polymers that knock down, insert, or replace a disease-associated RNA (Figure 1). RNA therapeutics act via diverse biological mechanisms, including antisense oligonucleotides (ASOs), RNA interference oligonucleotides, messenger RNAs (mRNAs), and single-guide RNA (sgRNA)/CRISPR systems. Some RNA therapeutics have already reached clinical trials and have been approved by the FDA (Table 1). For instance, Eteplirsan, a 30-nucleotide long phosphorodiamidate morpholino oligomer (PMO), is a splice switching oligo (SSO) that excises exon 51 in dystrophin RNA. The excision results in production of a functional dystrophin gene in duchenne muscular dystrophy (DMD) patients.¹ Improved symptoms, however, were observed in only 16% of patients. The specific challenges that Eteplirsan faced were low efficacy and rapid clearance of the PMO.^{2,3}

Very recently, the small interfering (siRNA) therapeutic, Patisiran, became the first FDA-approved siRNA therapy for hereditary transthyretin-mediated familial amyloidosis. It contains several 2' OMe modifications on the uridine nucleotides. Patisiran contains a lipid nanoparticle (LNP) formulation, which consists of a pH-sensitive fusogenic amino lipid (MC3), phosphatidylcholine (DSPC), cholesterol, and dimyristolglycerol-PEG.⁴ In a phase III clinical trial, Patisiran, administered intravenously, showed high therapeutic activity with no apparent side effects. Serum levels of transthyretin were 75% lower in patients treated with the drug compared to the placebo group.⁵ Lumasiran (ALN-GO1) is another promising siRNA therapeutic that reached clinical trials, developed for treatment of primary hyperoxaluria (PH1). In Lumasiran, the RNA drug is conjugated to *N*-acetylgalactosamine (GalNAc), and it targets glycolate oxidase in human hepatocytes. Recently conducted phase I/II clinical trial studies reported a 75% decrease in urine excretion of oxalates.

Excitingly, RNA therapy has the potential to provide a treatment option for multiple genetic diseases. Nevertheless, there are challenges with RNA stability, intracellular delivery, and off-target effects in vivo. In comparison to antibodies, that can only bind receptors on the cell surface to reach their cellular target, RNA therapeutics must cross cellular membranes and reach the desired intracellular compartment. Nuclease degradation, poor cellular uptake, and a low binding affinity to complementary target sequences are issues that need to be addressed. Due to the evolutionarily conserved viral defense pathway that is built into mammalian cells, especially immune cells, an innate immune system might be activated by exogenous RNA. This immune activation is driven by recognition of specific molecular patterns associated with pathogens by pattern-recognition receptors (PRRs), which act as RNA sensors. These RNA sensors, e.g., toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I), are located in intracellular compartments, such as the endosome and cytosol, respectively.⁶ In order to reduce immunogenicity while improving biodistribution and pharmacokinetic properties, chemical modifications of RNA can be introduced in the phosphodiester linkages, nucleobases, and/or ribose backbone.⁷ Furthermore, to address kidney filtration and to improve delivery, chemical conjugation and nanoparticle (NP)-based delivery methods can be applied.

To date, various NPs and nanomaterials (Figure 2) have been proposed for the delivery of therapeutic RNA.^{8–10} To mention a few, organic polymers, carbohydrate, and peptide-based formulations have been prepared and tested (Figure 2). LNPs and their modifications are among those systems that

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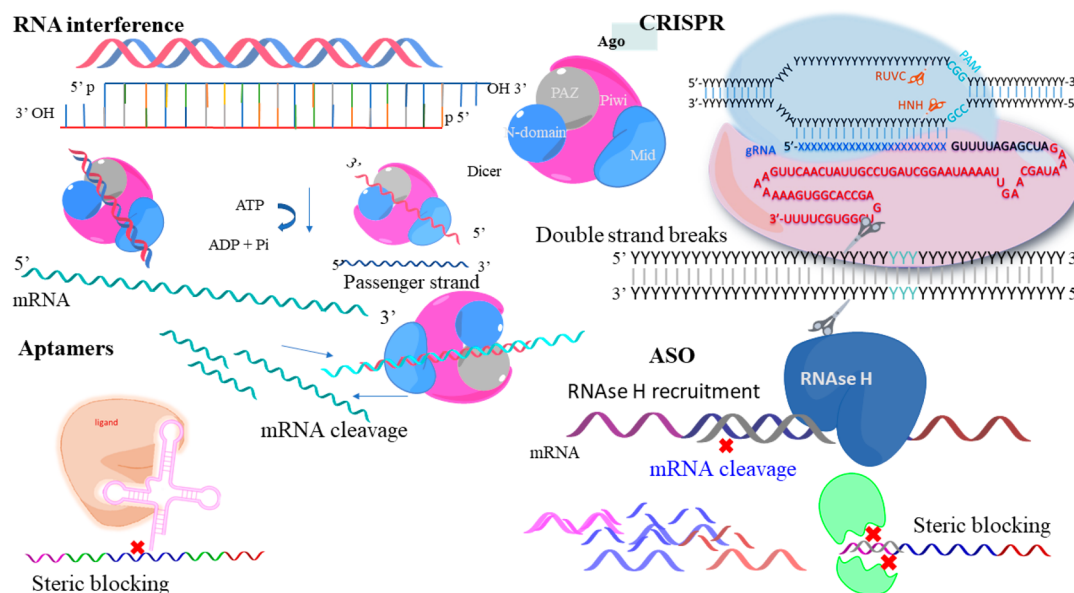


Figure 1. RNA therapeutics mechanism of action. (Left) RNA interference and aptamers; (right) CRISPR and ASOs.

Table 1. List of FDA-Approved RNA Therapeutics⁴⁴

name	RNA drug type	target site
Mipomersen, 2013	AS 20nt PS 2' MOE gapmer	apoB mRNA in homozygous familial hypercholesterolemia
Exondys 51, 2016	30nt PMO	DMD
Defibrotide	9–80nt, 90% ss ON, 10% ds; from pig's intestinal mucosa	liver veno-occlusive disease
Spinraza, Nusinersen	18nt PS 2'-O-methoxyethoxy ASO, all cytidines have methyl modification at 5' end	inclusion of exon 7 in spinal muscular atrophy mRNA
Vitraven, Fomivirsen, 1998 (discontinued by Novartis, 2006)	21nt PS	cytomegalovirus (CMV) retinitis
Macugen, 2004	2' OMe and the pyrimidine ribose sugars all 2'-F	VEGF1656, pM range affinity binding, macular degeneration
Patisiran, 2018	siRNA, 2' OME, lipid NP delivery	tanthyretin in hereditary transthyretin amyloidosis
Tegsedi (Inotersen), 2018	20nt, AS, 2' MOE RNA, PS, all cytidines have methyl modification at 5' end	transthyretin in hereditary transthyretin amyloidosis

⁴⁴ON, oligonucleotide; AS, antisense; PS, phosphorothioate; and PMO, phosphomorpholidate.

have been recently approved by FDA and therefore represent an extremely attractive object of studies.^{11–13}

In this review, we present the most recent delivery strategies for RNA gene therapeutics with a focus on emerging LNP solutions. Our focus is being paid to formulations in clinical translation, their unique features, challenges with preparation and screening, and ways to promote the development of new RNA LNP formulations.

■ LIPID NANOPARTICLES IN RNA THERAPY

Charged Cationic Lipids. LNPs gained much attention in the field of nucleic acid delivery when Felgner and colleagues, in 1987, demonstrated that cationic lipids, 1,2-di-*O*-octadecyl-3-trimethylammonium propane (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE), when formulated with pDNA, resulted in the formation of liposomes capable of *in vitro* transfection.¹¹ Only a couple years later, in 1989, DOTMA and DOPE were used to complex with luciferase mRNA to form LNPs for transfection of human, mouse, rat, *Drosophila*, and *Xenopus* cells.¹²

LNP formation with nucleic acids in an aqueous environment is driven by a process of self-assembly, which is influenced by the degree of hydrophilicity and hydrophobicity

within the regions of the lipid components. Cellular membranes, consisting of phospholipids in a lamellar or bilayer assembly, represent a classical example of lipid self-assembly occurring *in vivo*.¹⁴ In order to improve intracellular delivery of nucleic acids with LNPs, a transition from lamellar to the reversed hexagonal phase of the self-assembled lipid complex is required.¹⁵ The transition induces cell membrane destabilization, which is necessary for internalization of the cationic lipid–nucleic acid complex into the cytosol.¹⁵

Inspired by initial success, extensive effort has been put into the synthesis of cationic lipids for use in gene delivery both *in vitro* and *in vivo*. This has outlined key requirements in the structural design and properties of the cationic lipid, a positively charged headgroup (monocation or polycation, linear or heterocyclic) attached, via a linker bond, to a hydrophobic group (cholesterol or aliphatic).¹³ Several subtypes of cationic lipids exist, including monovalent and multivalent aliphatic lipids and cholesterol derivatives. The lipid structure, i.e., nature of the charged headgroup (primary, secondary, and tertiary amine or quaternary ammonium salt), is a critical determinant of transfection efficiency and the associated cytotoxicity.¹⁶ For instance, cationic derivatives of

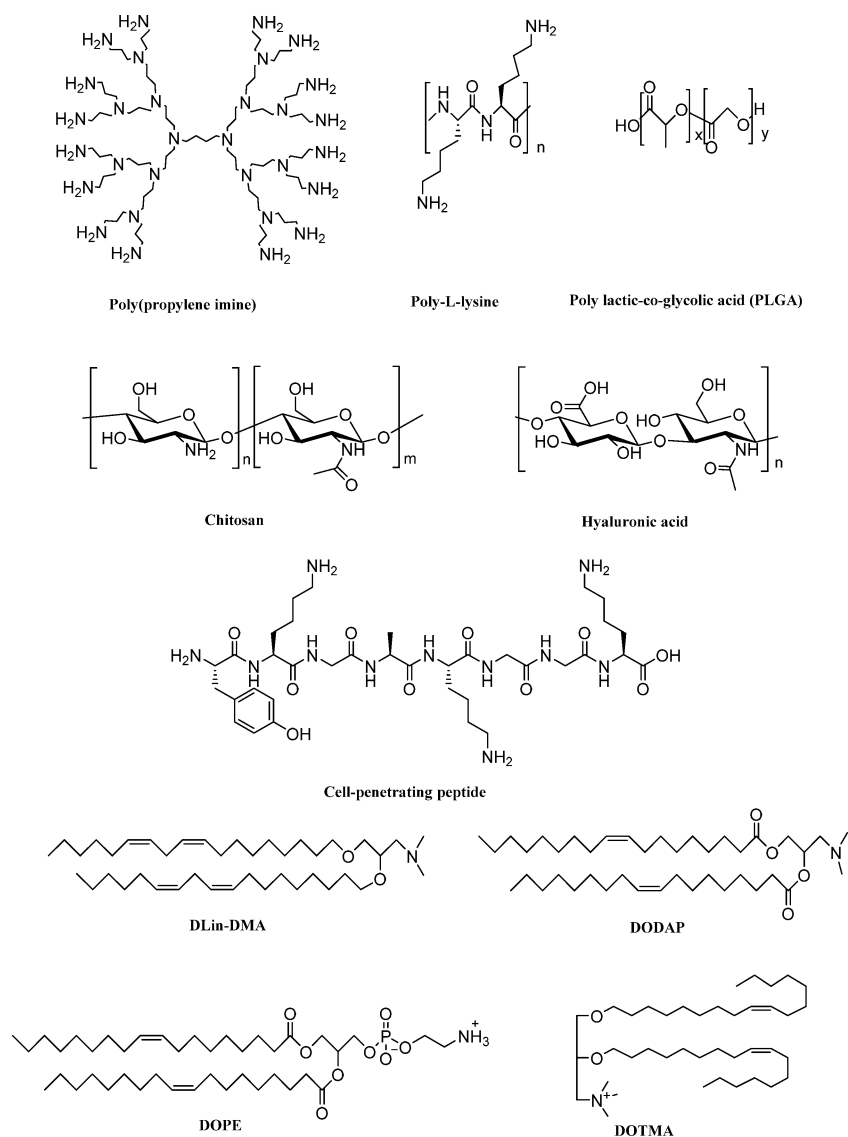


Figure 2. Examples of NP constituent chemical structures of polymers (poly(propylene imine), poly-L-lysine, and PLGA), carbohydrates (chitosan and hyaluronic acid), cell penetrating peptide, and lipids (DLin-DMA, DODAP, and DOPE).^{8–15}

cholesterol with quaternary head groups have been reported to display higher toxicity than their tertiary counterparts.¹⁷

The transfection efficiency of cationic lipids depends on several factors, such as the ability to complex with nucleic acids, to promote cellular uptake, and successive endosomal escape. It was later hypothesized, that, following endocytosis, an interaction between cationic lipids of the liposome and anionic phospholipids in the endosomal membrane promotes membrane disruption and subsequent release of LNP cargo into the cytosol.¹⁸ The length of the hydrophobic anchor or aliphatic chain as well as the degree of saturation also influence transfection efficiency and cytotoxicity. It has been reported, that, in a homologous series of lipids with alkyl chains spanning from C₁₄ to C₁₈, the shorter chain resulted in a bilayer with increased fluidity, which enhanced intermembrane mixing and the subsequent transfection process.^{13,19} Intermembrane mixing, driven by membrane destabilization, is dependent on the transition temperature of the lipids in the lipoplex. A lower transition temperature indicates that lipids will more readily shift from the high stability lamellar phase to the low stability hexagonal phase.²⁰ Unsaturated alkyl chains (i.e., oleoyl-based

lipids) have proven to be the most effective for gene delivery, with double-chained lipids being predominant in LNP investigations, as they are able to form lamellar phases without the need of helper lipids.²¹ The linker group typically consists of amide, ester, or ether bonds connecting the hydrophobic and hydrophilic regions of the lipid. Ether bonds are stable with high transfection efficiency; however, they also display toxicity. Ester bonds are biodegradable with reduced toxicity but can result in the premature release of cargo due to lipases and/or nucleases present in the endosome and lysosome, respectively.²² Additionally, carbamate-based linkers that show stability in circulation are hydrolyzed in the acidic endosomal compartment and are associated with reduced cytotoxicity.²³

There are several commercial products built on the success of cationic lipids, which have been applied as transfection reagents. The first transfection reagent for DNA in mammalian cells, lipofectin, consisted of DOTMA/DOPE.¹¹ The use of lipid-mediated transfection reagents, i.e. lipofectamine reagents, is now accepted as the gold standard for delivery of exogenous DNA or RNA into cells, despite their well-known cytotoxicity.²⁴ The inclusion of amphiphilic lipid molecules,

such as cationic lipids, in LNP formulations has proven to be an effective means of RNA encapsulation, cytoplasmic delivery, and endosomal escape. LNPs are hence regarded as one of the principle strategies for nonviral gene delivery,²⁵ with several formulations progressing into clinical trials.^{26,27}

Toxicity of LNPs and Strategies To Overcome It. A major drawback with the use of cationic lipids for gene delivery is the high net positive charge associated with the headgroup, as well as induction of immune response and short circulation time due to rapid plasma clearance.^{28,29} Furthermore, particles of cationic nature are known to undergo accumulation in the liver, lung, and spleen.³⁰ Lipoplexes, i.e., lipid–nucleic acid complexes, formulated with cationic lipids have been reported to induce inflammatory responses and immune cell activation following systemic administration, and the majority are endocytosed by Kupffer cells in the liver.³¹ Consequently, a great effort has been focused on the rational design of lipids with reduced toxicity for application in nucleic acid delivery. Nevertheless, even with the emergence of a vast variety of their analogues for gene therapy,³² gene expression was still associated with a substantial degree of cytotoxicity.³³

In general, toxicity is a combination of biochemical processes that leads to inflammation and short-term³⁴ or long-term³⁵ effects in an exposed organism. Toxicity of a low to moderate level can be beneficial to the treatment. Kranz et al. studied the immunological effects of intravenously administered RNA lipoplexes.³⁶ RNA lipoplexes trigger interferon- α release by dendritic cells and macrophages that are similar to the response of an early viral infection. In this case, interferon- α speeds up T cell maturation, which is beneficial for tumor treatment using RNA lipoplexes as a vaccine.³⁶ Moreover, combinational treatment by mRNA-LNP and FDA-approved TLR agonist, monophosphoryl lipid A, allowed for high antigen expression with controlled interferon release, showing a path for even safer strategies to induce T cell immunity toward cancer.³⁷

Nevertheless, toxicity due to uncontrolled cytokine release can be dangerous to a patient. Cytokine release syndrome (CRS) is a life-threatening toxicity that is caused by uncontrollably increased levels of pro-inflammatory cytokines, e.g., interleukin-6 and interferon.³⁵ CRS is rated in levels one to four, four being the hardest. It is considered life-threatening at levels three and four.³⁵ Being a huge concern in cancer immunotherapy, CRS of moderate to high levels is also observed as a result of LNP treatments. Hirsova et al. reported liver inflammation as a result of CRS after the treatment with palmitate and other lipids used as components of LNPs and lipoplexes.³⁸ Kubota et al. showed that inflammatory cytokine response differs among lipoplexes and LNPs.³⁹ siRNA-loaded LNPs released lower amounts of tumor necrosis factor α and interleukin-1 β than lipoplexes. The authors hypothesize that molecular structure has an impact on immune stimulation by NPs and suggest careful optimization of the composition prior to extended studies in vivo.³⁹

Complement activation is another pathological process that has been observed for LNP-formulated modified mRNA in rats and monkeys.⁴⁰ The authors point on the crucial importance of dose adjustment to make the complement activation mild and reversible. Besides that, coagulation parameters, cell count, and heart tissue might be affected by treatment with LNPs.⁴⁰ The use of shielding lipids, such as lipid-anchored polyethylene glycol (PEG) in LNP formulations, has been generally applied to increase systemic circulation time, reduce nonspecific cell

interaction and uptake, reduce particle size, and prevent aggregation during storage.^{41–43} However, there have been multiple reports that PEG induces production of anti-PEG immunoglobulin M (IgM) and subsequent complement activation, resulting in accelerated clearance.^{44,45} It has been reported that PEG shielding may reduce efficacy both in vitro and in vivo, due to steric blocking of the LNP–endosomal membrane interaction, hindering endosomal escape.⁴² Strategies to improve efficacy of PEGylated LNP include incorporation of acid- or pH-sensitive-modified PEG to promote the release of PEG from the lipid core, thereby reducing the negative effects of shielding on endosomal release.^{43,46}

Multiple works have been dedicated to reducing pro-inflammatory activity of nonvaccine RNA LNPs. Abrams et al. report on successful *Ssb* gene silencing in mouse liver by the siRNA-LNP drug candidate LNP201.⁴⁷ LNP201 induced an inflammatory response in mice, via activation of the MAPK kinase pathway. Notably, inflammation was completely inhibited using a glucocorticoid agonist, dexamethasone, without reducing the activity of the siRNA payload. In another work, an increased transfection efficiency and a reduction in cytotoxicity could be obtained when utilizing cholesterol analogs (DC-cholesterol) and helper/fusogenic lipids (DOPE) in LNP-mediated mRNA delivery.⁴⁸ Lastly, Asai and Oku³⁴ point on PEGylation as a steric block to pro-inflammatory interaction of LNP with immune cells. Besides PEG, other ligands, including peptides and antibodies, are suggested to decorate LNPs' surface, to overcome the systemic toxicity.³⁴

Ionizable Cationic Lipids. The off-target effect and systemic toxicity associated with the use of permanently charged cationic lipids, for RNA delivery in vivo, lead to the development of ionizable lipids with reduced toxicity and immunogenicity. 1,2-Dioleoyl-3-dimethylaminopropane (DODAP) was the first ionizable lipid utilized in a LNP formulation. Using DODAP, up to 70% encapsulation of DNA/RNA has been achieved, in both uni- and multilamellar liposomes.⁴⁹ This structure, consisting of two oleyl chains, has served as the foundation for the development of additional ionizable lipids, exemplified by Figure 2. 1,2-Dilinoleoyloxy-*N,N*-dimethyl-3-aminopropane (DLinDMA), another first generation ionizable lipid, with linoleyl hydrocarbon chains, was reported to be optimal for RNA delivery in hepatocytes, with up to 90% silencing of mRNA in hepatocytes in cynomolgus monkeys.⁵⁰ DLinDMA has since demonstrated initial proof of concept in humans⁵¹ and has resulted in the development of second generation ionizable cationic lipids, such as DLin-MC3-DMA. MC3-DMA, one of the lipid components in the Patisiran formulation, has been synthesized containing ester linkages for biodegradability. The biocleavable linker facilitates favorable stability at physiological pH while allowing enzymatic hydrolysis in tissues and intracellular compartments, due to local esterase and/or lipase activity.⁵² This promotes improved tolerability and a safety profile, while maintaining high potency in rodents and nonhuman primates (NHP).⁵² It should be noted, that the level of gene silencing in NHP was less than the level of gene silencing in mice. This may, in part, be because LNP composition was optimized in mice. Further optimization of the formulation composition would be required to attain optimal efficacy in NHP. These novel amino lipids, however, are the first demonstration of biodegradable lipids with an efficacy comparable to the most

advanced lipids currently available for siRNA delivery, and they show promise for use in future RNAi therapies.

A recent delivery platform combines a lipid component with unlocked nucleic-acid-modified RNA (LUNAR). Similarly to Patisiran, LUNAR achieves biodegradability due to the presence of an ionizable lipid with ester linkage in the lipid backbone.^{53,54} The hyperactive factor IX (FIX) mRNA variants used in the LUNAR system were reported to have 8–10 times the therapeutic effect compared to the current recombinant human FIX protein therapy. The authors reason that LUNAR-encapsulated FIX mRNA is preferentially targeted to the liver, translated into protein by hepatocytes, and released into circulation. The term “targeting” should, however, be used with caution, as the liver is a natural site of NP accumulation following intravenous administration.⁵⁵ That being said, the liver is a site for many physiological functions and a relevant target for many genetic diseases. So, this phenomenon may be harnessed for hepatic delivery strategies. Additionally, a combined approach using an ionizable lipid-like material (C12-200) formulated with helper lipids for delivery of Cas9 mRNA, and an AAV encoding a sgRNA and repair template, has been utilized for hepatocyte gene editing in vivo.⁵⁶ The combined viral and nonviral mediated delivery allowed for short-term expression of Cas9 nuclease, providing on-target gene editing while reducing off-target editing. Another ionizable lipid (8-O14B), with bioreducible properties, was used for the codelivery of supercharged Cre recombinase protein and Cas9:sgRNA both in vitro and in vivo.⁵⁷ The bioreducible nature is provided by the inclusion of a disulfide bond in the hydrophobic tail. This undergoes reduction in intracellular compartments due to, for example, the presence of high concentrations of glutathione, ultimately, facilitating endosomal escape of the protein-RNA complex. In human cell culture, the 8-O14B lipid enabled up to 70% Cre- and Cas9:sgRNA-mediated gene recombination and knockout. Under optimized in vitro conditions, the Cre/8-O14B complex was injected into different sites in the brain of mice. Notably, the nanocomplexes delivered to the brain were confined to the injection site. This may indicate potential for use in genome editing in specific neuronal populations.

A novel class of synthetic charge-unbalanced amino-lipids, termed cationic quaternary ammonium sulfonamide amino lipids (CSALs), have recently been explored for siRNA delivery.⁵⁸ Through synthesis of multiple lipid analogues with varied linker amine, aliphatic tail side chain, and headgroup amine, a lead CSAL LNP was developed. This CSAL LNP enabled in vivo delivery of an RNA drug candidate to the lungs in normal and tumor-burdened mice.⁵⁸ The systematic, modular design implemented here, for the library generation of CSALs, enabled the assessment of structural modifications and the relative contributions to biophysical properties of the LNPs in regard to size, surface charge, and siRNA encapsulation.

Structure and morphology of ionizable cationic lipids are being actively optimized, leading to several successful formulations for both siRNA and mRNA delivery. A phase I clinical trial is currently ongoing for the treatment of advanced solid tumors, whereby siRNA against EphA2 is delivered via an LNP (NCT01591356, Table 2). Another clinical trial involving delivery using an LNP, more specifically, an ASO encapsulated in an LNP (liposomal Grb2), is currently in phase I/II for the treatment of chronic myelogenous leukemia (CML)

Table 2. Current Clinical Trials Involving NP-Mediated Oligonucleotide Delivery

drug name	oligonucleotide	target	disease	delivery vehicle	phase	ClinicalTrials.gov identifier
STP705	siRNA	TGF-1 β and Cox-2	hypertrophic scarring	peptide (histidine–lysine copolymer)	I/II	NCT02956317
siG12D-LODER	siRNA	KRASG12D	cancer (pancreatic)	degradable polymeric matrix	II	NCT01676259
iExosomes ^b	siRNA	KRASG12D mutation	cancer (pancreatic)	extracellular vesicle (exosome)	I	NCT03608631
NU-0129	siRNA	BCL2L12	cancer (glioblastoma multiforme)	gold NP	I	NCT03020017
liposomal Grb2	ASO	Grb2 gene	cancer (CML)	LNP	Ib/IIa	NCT02923986, NCT02781883, NCT01159028
siRNA-EphA2-DOPC	siRNA	EphA2 gene	cancer (advanced solid tumors)	LNP	I	NCT01591356
Lipo-MERIT	mRNA	tumor-associated antigens	cancer (melanoma)	LNP	I	NCT02410733
TNBC-MERIT	mRNA	tumor-associated antigens	cancer (triple negative breast)	LNP	I	NCT02316457
mRNA-1325	mRNA	viral antigenic proteins	zika	LNP	I	NCT03014089
mRNA-2416 ^c	mRNA	OX40L	cancer (advanced/refractory solid tumor malignancies or lymphoma)	LNP	I	NCT03323398
Pbi-siRNA	shRNA	EWS/FLI1 gene	cancer (Ewing's sarcoma)	LNP	I	NCT02736565
MTL-CEBPA	saRNA	CEBPA gene	cancer (liver)	LNP	I	NCT02716012
SGT-53	pDNA	p53 gene	cancer (relapsed/refractory solid tumors)	LNP	I/II	NCT02354547, NCT02340156, NCT02340117

^aDOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; saRNA, small-activating RNA; shRNA, short hairpin RNA; and pDNA, plasmid DNA. ^bPhase I completed October 29th, 2018. ^cPhase I completed December 31st, 2018.

(NCT02923986, NCT02781883, and NCT01159028; Table 2).

LNP Structure–Function Relationship and Screening.

An universal delivery system applicable for the transfection of different classes of nucleic acids, i.e., DNA, siRNA, and mRNA, into different cell lines or primary cells has yet to be achieved. This is, in part, due to the varying nature of the nucleic acid therapeutics. For example, single/double-stranded DNA vs double-stranded RNA, mRNA, and CRISPR-Cas9 sgRNA all have different molecular masses. This means that NP systems are typically adapted to molecular features of the specific nucleic acid.⁵⁹ Moreover, the combinatorial chemical space of the LNP realm is immense. The sheer number of different lipids and lipid-like materials that can potentially be utilized for oligonucleotide delivery makes the screening for formulation parameters extremely laborious. Thus, it is difficult to fully explore the chemical space to find the optimal formulation. LNP formulation parameters can be systematically optimized through the one-factor-at-a-time (OFAT) method to generate lipid libraries for functional assessment. For example, to assess the efficiency of delivery *in vitro/in vivo* in order to reduce the toxicity associated with LNPs.⁶⁰ The libraries are rationally designed with varying lipid components and molar composition for the given therapeutic application and oligonucleotide cargo. Such a technique has been applied to optimize the LNP formulation for mRNA delivery in cancer immunotherapy.⁶¹ On the basis of the structure–function knowledge, a general guide for screening of LNPs has been described by Patel et al., who suggested the following screening steps, (1) selection of individual lipid components and preliminary screening of a formulation, (2) studying partitioning behavior and selecting the size of LNP, (3) assessing properties of combined LNPs *in vitro*, and (4) optimization.⁶² Although the initial design is well described, two key steps are missing in the workflow proposed by Patel et al., structural investigations of LNPs and *in vivo* assays.

Despite attempts to the rational design of LNP structure, LNP-mediated RNA therapies are limited by the poor understanding of how LNP structure and morphology influences biodistribution to off-target organs and delivery efficiency to target cells, *in vivo*. To improve this, structures of siRNA- and mRNA-loaded LNPs have been studied by dynamic solid-state nuclear magnetic resonance (NMR).⁶³ The LNPs were composed of the aforementioned ionizable cationic lipid DLin-MC3-DMA, a phospholipid DSPC, cholesterol, and DMPE-PEG2000. Phosphorus and carbon chemical shifts were found to be useful in determining different content and morphology of LNPs. A striking finding was that the structure of LNP was dramatically changed when the RNA payload was applied. Specifically, the loaded LNPs tended to result in layering of DSPC and DMPE-PEG around a rather homogeneous core. RNA cargo was located in the subcore or on the surface of LNP, depending on the composition.⁶³ This study introduces a new model for LNP structure (homogenous core–shell) and potentially rules out the multilamellar vesicle model, which has previously been used to describe LNPs. This highlights the need for further characterization techniques, such as solid-state NMR, to elucidate a better mechanistic understanding of RNA encapsulation by LNPs, the resulting supramolecular assembly, and the subsequent biological interactions in order to assist the rational design of future LNPs.

PEGylation, as previously mentioned, is a broadly applied modification of LNPs that improves biodistribution and reduces toxicity. Hence, the properties of PEG modification are important factors that need to be taken into consideration when preparing PEGylated LNPs. Wilson et al. used pulsed gradient spin echo (PGSE) NMR to investigate PEG shedding from LNPs *ex vivo*.⁶⁴ In doing this, a combination of DLin-MC3-DMA⁶³ with DOleylDMA or DSA/DMA-PEG was used. LNPs modified with PEG via shorter lipid anchors showed rapid shedding of PEG in rat serum (half-life time $t_{1/2}$ 0.64 h). Interestingly, extending the lipid chain from C14 to C18 prolonged the $t_{1/2}$ for PEG on the LNP surface up to 4.03 h.⁶⁴ This novel NMR method provides a way of studying the dynamics of PEG shedding *ex vivo*, allowing predictions of particle behavior *in vivo*. This will facilitate further understanding of the effect of structural variations in PEG-lipids as well as variations to the particle compositions, without the need for animal experiments. Extension of this method into more biologically relevant conditions would be required. It must be noted, that upon intravenous injection of LNPs, it would be expected that PEG shedding is more rapid due to the increased biological milieu.

Predictable *in vivo* RNA delivery is another highly desired structure–function correlation to be established for LNPs.^{65,66} Whitehead et al. studied a large library of 1400 biodegradable lipidoids as potent carriers for siRNA *in vivo*.⁶⁵ Prior to *in vivo* experiments, an extended *in vitro* screening of siRNA delivery by LNPs was conducted. The study was carried out in HeLa cells expressing two reporter proteins, firefly and Renilla luciferase. Reduced luciferase activity indicated toxicity associated with the LNP, those of which were not considered for further studies. Of the entire library, 82 LNP formulations were found to be highly toxic, reducing luciferase activity by over 50%. Next, according to an *in vitro* study, C12 and C13 fatty acids were abundant in the successful LNP population. Moreover, tertiary and secondary amines, alcohols, and branched or linear chains conferred efficacy, while ethers and rings did not, except amine-containing piperazine. The most potent LNPs successfully silenced Factor VII blood clotting factor and CD45 tyrosine phosphatase protein *in vivo*. These particles contained three or more C13 fatty acids per LNP, and over 50% contained a tertiary amine group in the lipid structure. A second-generation LNP library confirmed these efficacy criteria for siRNA delivery into human hepatocytes and immune cells.⁶⁵

Optimizing LNPs for mRNA delivery has been approached by Areta et al.⁶⁶ DLin-MC3-DMA-based LNPs were loaded with human erythropoietin mRNA, leading to NPs of 45 to 135 nm in diameter, corresponding to a DMPE-PEG content from 3 to 0.25 mol %, respectively. Cryo-TEM revealed details on the morphology of the LNPs. As above,⁶³ adding RNA shaped the LNP structure, leading to less structural variants than in the absence of the payload.⁶⁶ Rigid cylinder packing and nonspherical modality have been observed for the mRNA-LNPs in aqueous media. Phospholipid DSPC was located mainly on the surface, as in the previous NMR study.⁶³ LNP composition has been further optimized with regard to the DLin-cholesterol ratio, to achieve LNPs with a surface area per DSPC molecule of 1.2 nm². This resulted in maximum protein expression in human adipocytes and hepatocytes *in vitro*. LNPs with this surface area showed the highest protein production *in vitro*, especially at an NP size of 100 nm.⁶⁶ Regarding the optimal particle size of LNPs for RNA delivery,

there are contradictory results in the literature. Akinc et al. reported that in vivo efficacy of siRNA-lipidoid formulations increased with decreasing particle size.⁶⁷ In contrast, Bao et al. showed that the largest siRNA-LNPs resulted in the highest gene silencing activity in vivo.⁶⁸ More recently, Chen et al. identified siRNA-LNPs of 78 nm displaying the highest hepatic gene silencing in vivo.⁶⁹ This inconsistency, highlighted by Areta et al.⁶⁶ above, demonstrates that particle size alone may not be the only determinant for transfection efficiency. Rather, there is a dependence on particle size and particle surface composition. The latter being particularly prominent in the release of RNA from the endosomal compartment.

The work by Alabi et al.⁷⁰ brings in the pK_a as one of key determinants in LNPs fate in vivo. Indeed, only 5–10% RNA payload escape endosomes when special repercussions are not taken.^{65,66} Aiding endosomal escape by adjusting the pK_a of a phospholipid formulation is an important screening parameter that was proven to enhance siRNA delivery in vivo and in vitro.⁷⁰ Extensive structure–function assessment led to the realization that including ionizable cationic lipids, with an optimal pK_a 6.2–6.4, resulted in long-circulating liposomes, in vitro luciferase silencing in HeLa cells, and in vivo mouse factor VII silencing.^{70,71} The ionizable nature of the lipid allows for LNP formation with anionic RNA at low pH, where the lipids possess an overall cationic charge. The overall pK_a allows the lipid to remain deprotonated during circulation, reducing nonspecific cell interaction and subsequent early release of RNA cargo. While allowing protonation in the early or late endosome, which is necessary for facilitating membrane fusion and lipid mixing with the anionic lipids in the endosomal membrane.²⁰

There are three major aspects that are missing in many early works on LNPs which may have contributed to clinical trial terminations, (1) structure of LNPs, (2) correlation between in vitro and in vivo performance, and (3) delivery to a broad range of cells and tissues outside the liver, rather than focusing on the natural accumulation in hepatocytes. Novel strategies incorporate encoding elements into the LNP design to facilitate the simultaneous assessment of multiple factors in LNP structure and performance. Such an approach, pioneered by Dahlman et al., utilizes DNA oligonucleotide barcodes packaged within the LNPs to measure the biodistribution of distinct LNPs to different cells and tissues.^{72,73} Initially, Dahlman et al. chose to systematically vary three factors of one component in the LNP structure and their influence on biodistribution, the PEG tail length, PEG molecular weight (MW), and PEG mol.% in formulation, before proceeding to a larger study comparing the correlation between delivery efficiency in vitro to in vivo.^{72,73} The highlight of this study is that LNP delivery in vitro is a poor predictor of delivery in vivo, and as such, the gold standard in vitro screening approach needs to shift to in vivo screening. A further study by Dahlman et al. focused on screening for LNPs with functional mRNA delivery to nonliver tissues.⁷⁴ A discrepancy exists between biodistribution of LNPs and functional delivery of mRNA, as 96% of delivered RNA does not escape the endosome.^{75,76} This may vary with cell type or disease state. Hence, it becomes difficult to predict functional delivery based on particle biodistribution. Dahlman et al. chose to use a Cre-Lox system in mice, which allowed quantification of functional, cytosolic delivery of mRNA in vivo. Specifically, two LNPs (7C2 and 7C3) were identified out of >250, that efficiently deliver siRNA, sgRNA, and mRNA to endothelial cells.⁷⁴

There are still aspects missing from the screening strategies implemented here. For instance, the development and inclusion of high-throughput techniques for characterizing LNPs in terms of zeta-potential, pK_a , and lipid bilayer structure. The implementation of further characterization in the screening pipeline would facilitate advances in structure–activity relationships for nonliver tissues. These three studies highlight the necessity for an encoding system in high-throughput LNP assessment to elucidate fundamental understanding of the complex interplay between NP structural properties and delivery in vivo.

Tang et al. proposed a screening of the NP library with regard to interaction with immune cells as an early selection criteria for small molecules, to avoid toxicity in vivo.⁷⁷ Using an atherosclerosis model, 17 NP formulations were tested by the factor of inducing cholesterol efflux. Decorating NPs with protein APOA1 remarkably increased cholesterol efflux by the NPs. Besides this, phospholipid and core composition of NPs had an effect on the performance in vivo, with POPC dominant, 30 nm size spherical LNPs being the most effective and least toxic.⁷⁷ The authors hypothesize that particles with a small size combined with a long blood half-life promoted retention in atherosclerotic plaque macrophages. The fine-tuning of the LNP components and synthesis procedures improved the therapeutic index of a immunomodulatory molecule by favoring the delivery to aortic macrophages, rather than to splenic macrophages or the liver, which clear NPs from the blood and therefore reduce the bioavailability. This strategy of immunological screening using a combinatorial NP library may allow improvement in the precision of immunotherapies through tissue- and cell-specific delivery and the development of tailored nanotherapies for inflammatory diseases.

Delivery of LNP. Following an effective structure optimization, further improvement of the LNP can be achieved via surface decoration with specific ligands. The first generation of LNPs was limited to delivery via passive targeting, often utilizing the enhanced permeability and retention effect (EPR), a phenomenon occurring in solid tumors and infarcted areas associated with sites of inflammation and hypoxia.⁷⁸ Doxil, the first FDA-approved liposomal formulation of doxorubicin, incorporated a PEG coating to provide a steric shield for avoiding clearance by the reticuloendothelial system (RES) and allowing greater circulation time following intravenous administration.⁷⁹ The EPR effect is now considered the primary mechanism for the passive accumulation of NPs in tumors in vivo.⁵⁵ Likewise, the liver also accumulates NPs in a manner similar to the EPR effect. Once retained in the liver, NPs interact with hepatocytes, endothelial cells, B cells, and Kupffer cells. All of which become a major barrier when the objective is delivery to nonliver tissues or cells. A more desirable delivery approach would involve surface modification of the NP to promote delivery in a tissue or cell-specific manner in vivo. Improved delivery of LNPs would minimize nonspecific side effects (both on neighboring cells and systemically) and would reduce the nucleic acid payload.^{80,81} Preferential tissue/cell retention may be achieved by conjugating various targeting moieties to the NP surface. Initially, LNPs primarily utilized antibodies as the targeting moiety, due to their high specificity and availability.⁸² Since then, various other targeting moieties have been explored, including peptides, proteins, small molecule ligands, aptamers, antigen-binding (Fab) fragments, and single-chain variable

fragments (scFv).⁸³ The type of moiety is not the only determinant in targeting functionality, as the size, charge, density, and orientation also contribute to the overall efficacy.

The literature reports a mixture of responses from the use of ligands for LNP modification and their influence on biodistribution or pharmacokinetic profile. Some report no influence; whereas others suggest an improvement.⁸³ Nonetheless, receptor-mediated endocytosis using targeting ligands is the primary contributor to enhanced therapeutic response, via increasing internalization by target cells.^{84,85} A success story in antibody-mediated targeting has been reported in gene silencing, whereby liposomes containing CCR5 siRNA, decorated with lymphocyte function-associated antigen-1 (LFA-1) antibodies, reported delivery to T cells and macrophages and overall protection from HIV infection in mice.⁸⁶ However, there are considerations that need to be addressed with the use of LFA-1 antibodies for this approach, as leukocyte adhesion may be blocked and result in the silencing of pro-inflammatory molecules.⁸⁷ More recently, Ramshetti et al. reported specific binding, uptake, and silencing of CD45 in murine T lymphocytes following IV injection, using anti-CD4 mAb-targeted LNPs.⁸⁸ Even at a low dose, effective T cell silencing was observed in the blood, spleen, bone marrow, and inguinal lymph nodes.

For B cell malignancy, anti-CD38 mAb-modified LNPs achieved specific uptake in human mantle cell lymphoma cells (MCL) in the bone marrow of xenografted mice. In vitro studies demonstrated specific delivery of siRNA against cyclin D1 (siCycD1) to B cells. However, it must be noted, that gene silencing of CycD1 was not successfully demonstrated in a direct manner in vivo. Rather, an overall survival benefit was observed for mice treated with the anti-CD38-LNP-siCycD1.⁸⁹ Moreover, LNPs have been functionalized with hyaluronan (HA), a natural ligand for the CD44 receptor. CD44 is overexpressed on the surface of multiple cancer cell types. HA-decorated LNPs delivered siRNA cargo locally to glioblastoma multiforme (GBM) cells in a murine xenograft model, significantly prolonging survival of treated mice.⁹⁰ In fact, this was the longest reported survival of mice in this type of GBM model and shows promise for the use of therapeutic siRNAs in localized treatments for GBM.

Returning to the functionality of ligands, their orientation on the LNP is a key factor. This is particularly relevant for monoclonal antibodies (mAb), where the accessibility of the Fab is required for full biofunctionality. It is becoming increasingly evident that conventional covalent immobilization techniques, e.g., using EDC/NHS, are ineffective at directional coupling, due to nonspecific interactions with multiple reactive sites on the protein.^{91,92} The lack of directionality in mAb immobilization has been addressed by Jeong et al., whereby orientation-controlled antibody conjugation was achieved using copper-free click chemistry.⁹³ A modular platform for targeted RNAi therapeutics has recently been developed by Kedmi et al., whereby LNPs are functionalized with targeting antibodies via a recombinant protein, named anchored secondary scFv enabling targeting (ASSET).⁹⁴ This platform has since been used for the cell-specific delivery of mRNA to leukocytes in mice, representing a flexible platform that may have great potential in precision medicine.⁹⁵

Nucleic acid aptamers offer several advantages over their antibody counterparts regarding targeting functionality. Aptamers are smaller, lack immunogenicity, have higher stability, and show versatility in chemical production and

modification.⁹⁶ Upon binding to cell-surface receptors, most aptamers undergo internalization, which makes them ideal targeting moieties for oligonucleotides, i.e., siRNA and miRNA. However, clinical development of therapeutic aptamers is still far behind that of monoclonal antibodies. A recent termination of a phase III clinical trial of an anticoagulant aptamer against factor IXa⁹⁷ exemplifies this and indicates that it will still be some time before clinically effective aptamers are developed for use in NP targeting.

Alternate examples of ligands for LNP decoration are GalNAc, which has a high affinity for the asialoglycoprotein receptor on hepatocytes;⁹⁸ ApoE lipoprotein, which facilitates receptor-mediated endocytosis into hepatocytes;⁹⁸ as well as folate and transferrin for targeting cancer cells.^{99,100} Recently, a novel targeted liposomal formulation, utilizing a folate-containing lipoconjugate (FC) and PEG spacer, has been investigated for nucleic acid delivery to folate receptor (FR)-expressing tumor cells.¹⁰¹ These liposomes were formed under low N/P conditions which favored a reduced cytotoxicity and resulted in enhanced transfection efficiency, both in vitro and in vivo, in comparison to the untargeted formulation. Furthermore, polypeptide pPB-modified stable nucleic acid lipid NPs (pPB-SNALPs) have been reported to selectively deliver siRNA against heat shock protein 47 (Hsp47) to the liver, which has shown efficacy for the targeted therapy of hepatic fibrosis.¹⁰² These modified SNALPs displayed increased uptake by hepatic stellate cells of mice in vitro and in vivo, indicating an effective liver-targeting delivery system. Again, the "liver-targeting" effect observed here is facilitated by the natural hepatic accumulation following intravenous administration. Nevertheless, the current antifibrotic drug treatment is ineffective at liver targeting, so there is promise for the use of modified nucleic acid-LNPs for hepatic fibrosis.

There is currently a bottleneck in the translation of nucleic acid-LNP therapies from clinical trials to products on the market, despite immense effort. Such therapies rely on the establishment of product safety, stability, and performance in vivo that has yet to be achieved. Further assessment is required to characterize and comprehend the particle physicochemical properties, in terms of composition, size, morphology, polydispersity, surface properties, and serum stability, and their biological fate.¹⁰³ The cytotoxicity and innate immune activation associated with the use of cationic LNPs has also represented a major barrier in the clinical translation. The utilization of ionizable and PEGylated lipids has to some extent addressed the issues of immunogenicity and mononuclear phagocyte system (MPS) clearance. However, studying the in vivo fate of particles relies on animal models which may not be so feasibly extrapolated to humans.²⁵ Additionally, production and formulation conditions, as well as storage conditions, are an essential factor in robust and reproducible manufacturing.²⁵

Toward Mass Production of Therapeutic LNP. When the LNP formulation has been optimized and tested in vitro and in vivo, the next preclinical step requires extended studies. To perform these studies, large amounts of RNA LNP drug candidate are needed. Currently, large-scale synthesis of LNPs is complicated and expensive. Synthesis optimization is therefore a crucial obstacle for RNA LNPs to be overcome in the upcoming years. Having on-hand a high quality, large scale RNA drug, short or long, and LNP formulations is an objective of ongoing work in both academia and industry. Today, up to 10 g of RNA synthesis can be performed.¹⁰⁴ The synthesis applies a solid-phase phosphoramidite chemistry,

which allows for controlled incorporation of additional modifications, such as LNA, 2'-OMe RNA, 2'-F-RNA, etc. GMP grade RNA LNPs can be further obtained in specialized synthesis units.^{105,106} Patisiran, the FDA approved siRNA therapeutic mentioned above, requires systemic administration at a dose of 0.3 mg/kg. Therapeutic mRNA, on the other hand, can be 100 times larger than a single siRNA duplex. mRNA production is currently carried out by a labor- and reagent-demanding cloning-expression approach.¹⁰⁷ Due to complicated, multistep preparation, the price of mRNA drug candidates is high, and the availability of certain modifications is limited. To address this issue, improved synthetic work flows have been explored.^{108,109}

Conventional methods of LNP production, e.g., lipid-film hydration and ethanol injection, despite issues in reproducibility and scalability, have, until recently, been the primary technique utilized.¹¹⁰ These drawbacks stimulated development of improved production strategies, based on the ethanol injection method. T-junction mixing for lipid-based drug delivery was pioneered in 1999 as a technique for production of DNA-lipoplexes, which provided a controlled mixing environment and resulted in reproducible production.¹¹¹ Since then, techniques utilizing microfluidic mixing have been used to encapsulate mRNA, siRNA, and pDNA in LNPs. This has revolutionized LNP production for scalability and reproducibility.¹¹² Microfluidic hydrodynamic focusing (MHF) and staggered herringbone mixing (SHM) represent the forefront in this technology.^{113,114} The controlled rapid mixing of two miscible phases, lipids in ethanol and nucleic acids in aqueous buffer, defined by the total flow rate (volume/time) and FRR (ratio of aqueous-to-organic flow rate) allow the resulting particle size and distribution (PDI) to be well-defined.¹¹⁵ Microfluidic chips may be designed so that the LNP morphology is predetermined and may be tailored to the application. Chips may also be parallelized in SHM to enable up-scaling of LNP production.^{41,116}

Fang et al. reported large-scale synthesis of lipid NPs using a multi-inlet vortex reactor (MIVR) (Figure 3).¹¹⁷ The MIVR device differs from previously reported microfluidic and sonication methods. It consists of either two or four radially symmetric inlets connected to circular reaction chamber. The device mixes an organic phase containing polymers and an aqueous phase, which acts as an antisolvent. Using the MIVR, up to 50 mL of LNP formulation, with a concentration of 2.5 mg/mL, can be produced only in one minute. The authors state that their methodology can be extended to a broad range of loaded LNPs, including RNA LNP, without dramatic reduction in product parameters.¹¹⁷

Kim et al. developed an approach for mass production of LNPs with a yield over 3 g per hour.¹¹⁸ This is approximately 1000 times faster than that of existing microfluidic devices. The principle behind the technique is based on creating symmetric microflow rates by 3D focusing of reagents in selected solvents, which occurs within a simple three-inlet chamber. The particle size can be readily controlled by adjusting the flow rate for the reagents. The reproducibility of the technique is also extraordinary, with a PDI < 0.1 being achieved in over an 100 g production scale.¹¹⁸

CONCLUSION AND FUTURE DIRECTIONS

There is great promise for the future development of LNPs for RNA therapeutics. The development of large-scale, reproducible production of LNPs and their payloads^{41,111–116} in

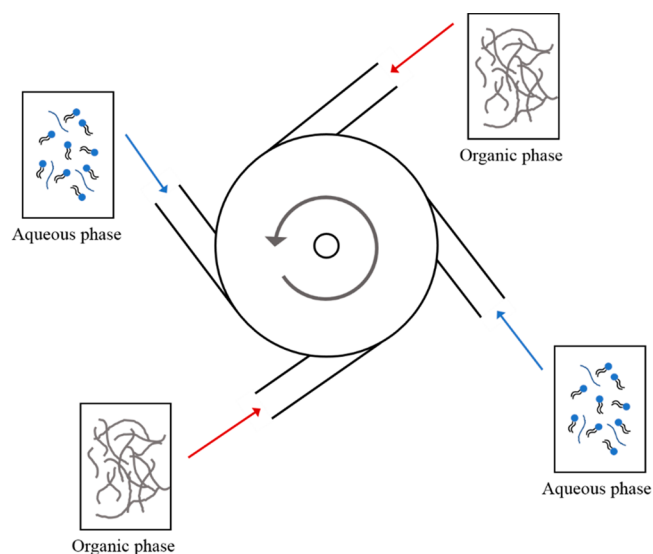


Figure 3. Diagrammatic working mechanism of an MIVR device for the synthesis of lipid–polymer hybrid NPs.¹¹⁷ The organic phase may contain dissolved polymer, and the aqueous phase may contain a lipid/lipid–PEG mixture. The four separate streams are directed into a circular reaction chamber which facilitates the self-assembly of NPs, which are collected from the outlet.

clinically relevant quantities could enable a shift of focus in drug development toward RNA therapeutics. Besides offering more material for trials, reducing the cost of RNA LNP drugs would make therapy more accessible to a larger group of patients. The incorporation of target-specific ligands into the LNP formulation, such as monoclonal antibodies or natural ligands, is expected to improve the safety profile of RNA therapeutics by reducing off-target effects and allowing cell-specific interaction, which has promoted the development of effective *in vivo* administration approaches. However, there is still a need to incorporate aspects of LNP functionalization into the synthesis process to allow reproducible preparation of targeted LNP formulations.

Recent developments in RNA-LNP therapeutics have been translated into several clinical trials, albeit with many challenges. Advances in high-throughput LNP screening and structural characterization approaches have broadened our understanding of the complex macromolecular interactions governing the delivery and efficacy of gene-therapeutics. However, the *in vivo* fate still needs further clarification for the full therapeutic potential of RNA oligonucleotides to be realized. RNA therapeutics has the potential to expand the range of druggable targets for multiple disease types and synergize with existing therapies to provide novel therapeutic approaches for currently untreatable diseases. In the field of cancer immunotherapy, which is currently dominated by antibodies, small molecules, and engineered T cells, there is still a need for efficacy improvement. CRISPR/Cas is a recently proposed gene editing mechanism that, in order to become an efficacious therapeutic tool, still requires an effective delivery system. Several reports apply LNPs for encapsulating components of the CRISPR/Cas complex, with already positive results achieved *in vivo*.^{56,57,119} Large off target effects and poor cellular uptake of CRISPR/Cas components are crucial issues to be addressed in the future. Personalized cancer vaccines are another emerging direction for RNA lipoplexes and RNA LNPs. Successful trials have been

performed for several RNA vaccines and have been brought into clinical testing, e.g., Lipo-MERIT and mRNA 2416. Developing more vaccines, both in a therapeutic or prophylactic sense, would be a way to approach cancer in a preventive way, which has not been possible up until now.

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ABBREVIATIONS

ApoE, apolipoprotein E; AS, antisense; ASO, antisense oligonucleotide; ASSET, anchored secondary scFv enabling targeting; Cas9, CRISPR-associated protein 9; CCR5, C–C chemokine receptor type 5; CD, cluster of differentiation; CML, chronic myelogenous leukemia; CRISPR, clustered regularly short palindromic repeats; CRS, cytokine release syndrome; CSAL, cationic quaternary ammonium sulfonamide amino lipids; DLinDMA, 1,2-dilinolexyloxy-*N,N*-dimethyl-3-aminopropane; DMD, duchenne muscular dystrophy; DMPE, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine; DNA, deoxyribonucleic acid; DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTMA, 1,2-di-*O*-octadecenyl-3-trimethylammonium propane; DSPC, phosphatidylcholine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EphA2, ephrin type-A receptor 2; EPR, enhanced permeability and retention effect; Fab, antigen-binding fragment; FR, folate receptor; GalNAc, *N*-acetylgalactosamine; HA, hyaluronan; Hsp47, heat shock protein 47; IgM, immunoglobulin M; LFA-1, lymphocyte function-associated antigen-1; LNP, lipid NP; LUNAR, lipid-enabled and unlocked nucleic-acid-modified RNA; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MCL, mantle cell lymphoma; MHF, microfluidic hydrodynamic focusing; MIVR, multi-inlet vortex reactor; mRNA, messenger RNA; NHS, *N*-hydroxysuccinimide; NHP, nonhuman primates; NMR, nuclear magnetic resonance; NP, nanoparticle; OFAT, one-factor-at-a-time; ON, oligonucleotide; PAM, polyamidoamine dendrimers; PDI, polydispersity index; PEG, polyethylene glycol; PEI, polyethyleneimine; PH1, primary hyperoxaluria 1; PLGA, poly(lactic-co-glycolic) acid; PMO, phosphorodiamidate morpholino oligomer; PRR, pattern-recognition receptors; PS, phosphorothioate; RES, reticuloendothelial system; RIG-I, retinoic acid-inducible gene I; RNA, ribonucleic acid; siCycD1, siRNA against cyclin D1; scFv, single-chain variable fragment; sgRNA, single-guide RNA; SHM, staggered herringbone mixing; siRNA, small interfering RNA; SNALP, stable nucleic acid lipid NPs; SSO, splice switching oligo; T cell, T

lymphocyte; TEM, transmission electron microscopy; TLR, toll-like receptor

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